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Authors

Verkman, AS

Solomon, AK

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Kinetics of Phloretin Binding to Phosphatidylcholine Vesicle Membranes

A. S. VERKMAN and A. K. SOLOMON

From the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The submillisecond kinetics for phloretin binding to unilamellar phosphatidylcholine (PC) vesicles was investigated using the temperature-jump technique. Spectrophotometric studies of the equilibrium binding performed at 328 nm demonstrated that phloretin binds to a single set of independent, equivalent sites on the vesicle with a dissociation constant of $8.0\ \mu\text{M}$ and a lipid/site ratio of 4.0. The temperature of the phloretin-vesicle solution was jumped by 4°C within $4\ \mu\text{s}$ producing a monoexponential, concentration-dependent relaxation process with time constants in the $30\text{--}200\text{-}\mu\text{s}$ time range. An analysis of the concentration dependence of relaxation time constants at pH 7.30 and 24°C yielded a binding rate constant of $2.7 \times 10^8\ \text{M}^{-1}\text{s}^{-1}$ and an unbinding constant of $2,900\ \text{s}^{-1}$; ~66 percent of total binding sites are exposed at the outer vesicle surface. The value of the binding rate constant and three additional observations suggest that the binding kinetics are diffusion limited. The phloretin analogue, naringenin, which has a diffusion coefficient similar to phloretin yet a dissociation constant equal to $24\ \mu\text{M}$, bound to PC vesicles with the same rate constant as phloretin did. In addition, the phloretin-PC system was studied in buffers made one to six times more viscous than water by addition of sucrose or glycerol to the buffer. The equilibrium affinity for phloretin binding to PC vesicles is independent of viscosity, yet the binding rate constant decreases with the expected dependence ($k_{\text{binding}} \propto 1/\text{viscosity}$) for diffusion-limited processes. Thus, the binding rate constant is not altered by differences in binding affinity, yet depends upon the diffusion coefficient in buffer. Finally, studies of the pH dependence of the binding rate constant showed a dependence ($k_{\text{binding}} \propto [1 + 10^{\text{pH}-\text{pK}}]$) consistent with the diffusion-limited binding of a weak acid.

INTRODUCTION

Phloretin markedly affects the transport of electrolytes and nonelectrolytes through biological and artificial membrane systems. In the human red blood cell, phloretin strongly inhibits hexose and chloride transport (LeFevre, 1961; Wieth et al., 1973); it also lowers the permeability of nonelectrolytes such as glycerol and urea (Macey and Farmer, 1970) and 2,3-butanediol (Owen and Solomon, 1972). In artificial bilayer membranes, phloretin increases carrier-mediated cation conductivity and decreases lipophilic anion conductivity (Anderson et al., 1976; Melnik et al., 1977). These observations suggested that

phloretin reduces the electrical potential of the membrane interior with respect to the aqueous phase. This reduction in potential could occur if phloretin, which has a dipole moment of 5.6 debye, orients at or near each membrane surface in a direction opposite to the intrinsic dipole potential of the membrane.

Jennings and Solomon (1976) showed that phloretin binds to sets of well-defined sites on a red blood cell membrane. They also demonstrated that phloretin crosses this membrane with a permeability coefficient of 2×10^{-4} cm/s at pH 7.0 and 22°C. In order to understand how phloretin acts in a membrane and is transported through it, we studied the equilibrium binding and binding kinetics of phloretin in an artificial membrane system. Phloretin was chosen for this study because of its extensive role in biological processes and because its acid-base properties make it especially suitable for binding studies using optical absorption. Phosphatidylcholine (PC) vesicles were chosen as the model lipid bilayer because they are single-walled, easily reproducible, closed vesicles that have been extensively characterized by Huang (1969).

The permeation of a molecule through a membrane has been modeled as a series of steps consisting of binding at one surface of a membrane, several jumps across the membrane and unbinding from the opposite side (Zwolinski et al., 1949; Träuble, 1971). We have measured equilibrium binding affinities and studied the submillisecond events of phloretin binding and unbinding using the temperature-jump technique. The goal of these experiments on the phloretin-PC vesicle system is to delineate the detailed microscopic processes that lead to the observed macroscopic permeability of phloretin in a model membrane system. This may reveal some basic mechanisms of nonelectrolyte transport through bilayer lipid membranes.

MATERIALS AND METHODS

Phloretin was purchased from ICN K & K Laboratories, Inc. (Plainview, N. Y.) and its purity was checked by thin-layer chromatography in chloroform-propanol (3:1). Naringenin was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. Phloretin and naringenin were added to buffers of the following composition: 0.1 M Tris (Fisher Scientific Co., Pittsburgh, Pa.) or 0.1 M Tris-maleate (Sigma) and 0.1 M KCl (Sigma) from a 0.1 M stock solution in ethanol. Buffers having different viscosities were prepared by dissolving sucrose (Fisher) or glycerol (Fisher) in water before adding vesicles and phloretin. Egg phosphatidylcholine (PC) was obtained from Makor Chemicals Ltd. (Jerusalem, Israel) and gave a single spot after thin-layer chromatography on Silica Gel G (ICN Nutritional Biochemicals, Cleveland, Ohio) using chloroform-methanol-water (65:25:4).

Vesicle Preparation

Unilamellar vesicles were prepared by sonication using the method of Huang and Thompson (1974). Lipid stored at -20°C in ethanol was spun in a rotary evaporator to remove ethanol, lyophilized, and suspended in buffer at a lipid concentration of ~25 mM. The suspension was sonicated at 4°C under N₂ for 1 h using a Branson model W 185 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). The vesicles were then centrifuged at 40,000 g for 45 min to remove titanium particles and lipid debris in a Sorvall model RC2-B refrigerated centrifuge (DuPont Instruments-

Sorvall, Newtown, Conn.). Vesicles were stored under N_2 at $5^\circ C$ and used within 1 wk of preparation. Lipid concentration was assayed by the method of Gomori (1942).

Binding Studies

Binding studies were performed using the semiautomatic titrator shown in Fig. 1. This device was used for optical absorption titrations and fitted onto a model 2220

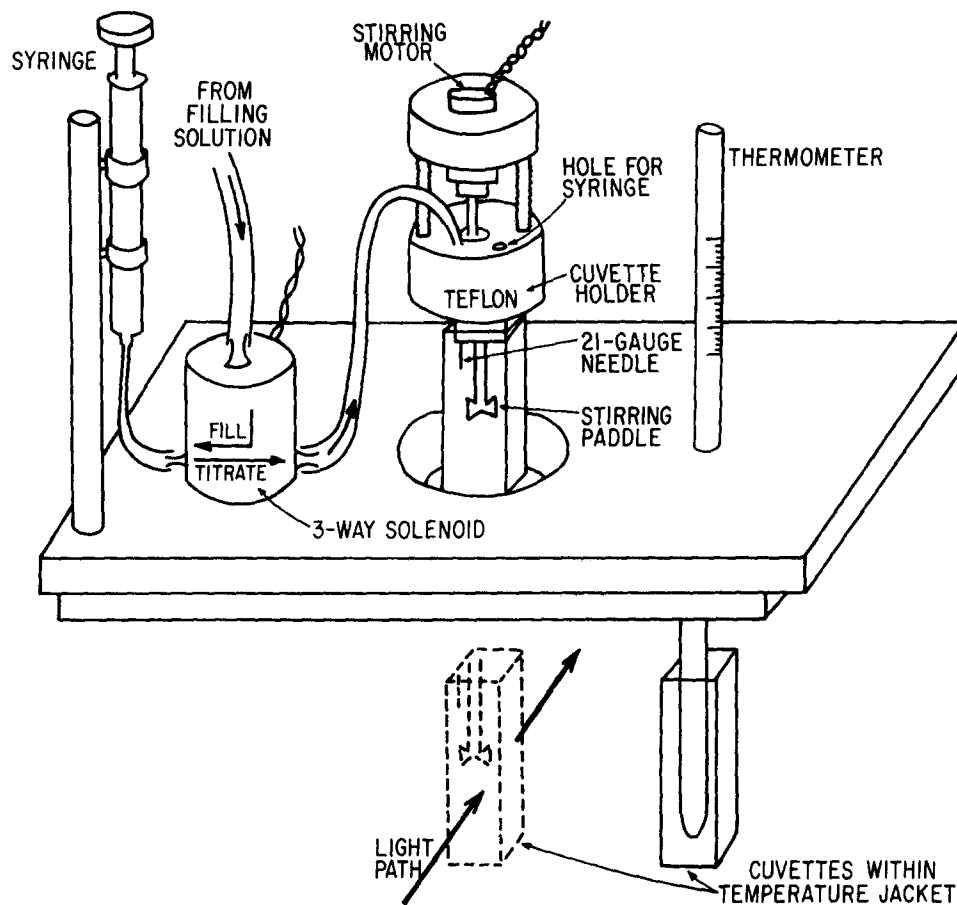


FIGURE 1. Semiautomatic titration device. Known aliquots of titrating solution were added using a hand-driven Hamilton syringe, which fills the cuvette through a 27-gauge needle. The three-way solenoid valve switched between the syringe filling conformation and syringe titrating conformation. The solution is constantly stirred using the paddle, and optical absorption measurements are made using the optics of a Beckman DU spectrophotometer.

adapter (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) of a Beckman model DU spectrophotometer (Beckman, Instruments, Fullerton, Calif.). A cuvette was held by a teflon connecting cap similar to the design of Kleinfeld et al. (1979), and the solution was constantly stirred by a paddle connected to a micro DC motor (15C11-104, Portescap U. S., New York).

In a typical titration 2 ml of buffer was added to the 4-ml quartz cuvette and the cuvette with connecting cap was lowered into the aluminum slab so that the light beam passed below the level of the propeller. The spectrophotometer was calibrated to read an optical density of zero and then microliter quantities of vesicles were added using a 10 μ l Hamilton syringe inserted into a hole which has been drilled into the teflon connecting cap. Then 200 ± 1 μ l aliquots of the titrating solution (for example, 250 μ M phloretin) was added to the cuvette repeatedly by manually filling and emptying the mounted Hamilton syringe through a three-way solenoid valve (General Valve Corp., model 3-151-900, E. Hanover, N. J.). The outflow from the valve passed into the cuvette via teflon tubing connected to a 27-gauge steel needle. The temperature was monitored using a thermometer placed in an identical cuvette lying within the same temperature-controlled jacket.

Duplicate titrations performed with this instrument were reproducible within 1 percent.

Temperature-Jump Studies

Temperature-jump studies as described by Eigen and DeMaeyer (1967) were performed on an apparatus designed by Verkman et al. which is described in detail elsewhere (Verkman et al., 1980). A 4°C temperature increment was obtained within 3 μ s by discharging a 0.1 μ F capacitor charged to 15,000 volts across a 0.8 ml solution volume. The instrument resolution time is ~ 5 μ s. The extent of phloretin binding was followed by optical absorption of light at 328 nm. The data were recorded on a waveform recorder (model 805, Biomation Corp., Cupertino, Calif.), which has a maximum sampling rate of 0.2 μ s per point. Data were then transferred to a PDP 11-34 computer (Digital Equipment Co., Marlboro, Mass.) for storage on disk and numerical analysis.

Calculations

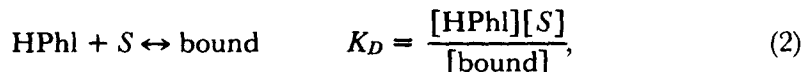
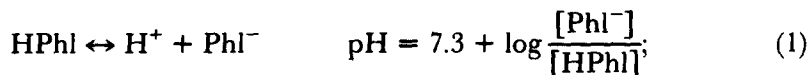
The data obtained from temperature-jump experiments were fitted to exponential functions by the nonlinear Newton's method as described in detail elsewhere (Verkman, 1979). The data from Figs. 2 b, 5, 6, and 7 were fitted to straight lines by the weighted least squares procedure. Standard deviations and correlation coefficients were calculated by usual procedures (Bevington, 1969).

RESULTS AND DISCUSSION

Equilibrium Binding Experiments

PHLORETIN The dissociation constant (K_D) and number of lipids per binding site (L/S) for the binding of phloretin to PC vesicles were determined by spectrophotometric titration. Phloretin is a weak acid ($pK_a = 7.3$) which has two absorption peaks in the ultraviolet region: a peak at 328 nm corresponding to its ionized form [Phl^-] and another peak at 280 nm corresponding to its un-ionized form [$HPhl$]. LeFevre and Marshall (1959) showed that a pH decrease caused an increase in phloretin binding by the red blood cell, which correlated well with the increased [$HPhl$] concentration. They interpreted this finding as preferential binding of the [$HPhl$] form by the red blood cell. Data from the present studies confirm that it is the [$HPhl$] form that binds to PC vesicles, and the binding is described using a single set of independent, equivalent binding sites which is consistent with exclusive binding of the un-ionized form of phloretin.

The acid-base and binding equilibria of phloretin are described by



where $[S]$ is the concentration of free vesicle sites and $[\text{bound}]$ is that of bound sites. If the total concentration of phloretin added is $[\text{Phl}]_{\text{tot}}$ and sites added is $[S]_{\text{tot}}$, then by conservation,

$$[\text{Phl}]_{\text{tot}} = [\text{HPhl}] + [\text{Phl}^-] + [\text{bound}] \\ = (1 + 10^{\text{pH}-7.3})[\text{HPhl}] + [\text{bound}]; \quad (3)$$

$$[S]_{\text{tot}} = [S] + [\text{bound}]. \quad (4)$$

Eqs. 2 and 4 taken together can be rewritten in the form of a double reciprocal plot in which a plot of $1/[\text{bound}]$ vs. $1/[\text{HPhl}]$ yields a straight line with slope $K_D/[S]_{\text{tot}}$ and intercept $1/[S]_{\text{tot}}$:

$$\frac{1}{[\text{bound}]} = \frac{K_D}{[S]_{\text{tot}}} \frac{1}{[\text{HPhl}]} + \frac{1}{[S]_{\text{tot}}}. \quad (5)$$

In the titrations performed, the pH was held constant by 0.1 M Tris buffer. Known amounts of total phloretin and lipid were added to a cuvette and the OD_{328} was measured. The concentration of $[\text{HPhl}]$ and $[\text{bound}]$ are deduced from Eqs. 3 and 6,

$$\text{OD}_{328} = \epsilon_{\text{free}} ([\text{HPhl}] + [\text{Phl}^-]) + \epsilon_{\text{bound}} [\text{bound}], \quad (6)$$

where ϵ_{free} and ϵ_{bound} are the extinction coefficients ($\text{OD}_{328}/\mu\text{M}$; 1 cm path) of the free and bound forms of phloretin, respectively. ϵ_{free} contains contributions from $[\text{HPhl}]$ and $[\text{Phl}^-]$ and was determined directly by measuring the OD_{328} of a known concentration of phloretin at a given pH. ϵ_{bound} was determined self-consistently as shown in Fig. 2 *a*. Curve α shows the fitted values for K_D determined from different trial values for ϵ_{bound} using Eqs. 5 and 6 for a single titration experiment. Curve β represents the same plot for another titration in which a higher lipid concentration was used. Line γ is a limit for $[S]_{\text{tot}} \gg [\text{HPhl}]_{\text{tot}}$,

$$K_D = \frac{[S]_{\text{tot}}(\psi - \epsilon_{\text{bound}})}{\epsilon_{\text{free}}(1 + 10^{\text{pH}-7.3})}, \quad (7)$$

where ψ is the OD per micromolar of phloretin as phloretin is added to a concentrated (3 mM) lipid solution ($\psi = \text{OD}_{328}/[\text{Phl}]_{\text{tot}}$). If the free form of phloretin did not absorb light, ϵ_{free} would equal zero and Eq. 7 would predict $\psi = \epsilon_{\text{bound}}$, as expected. All three curves in Fig. 2 *a* intersect at the true value of ϵ_{bound} which then uniquely determines K_D and the lipid per site ratio (L/S).

Fig. 2 *b* shows the reciprocal plot for phloretin binding to vesicles at pH 7.30 using $\epsilon_{\text{bound}} = 0.0016$ from Fig. 2 *a*. The factor $[S]_{\text{tot}}^0/[S]_{\text{tot}}$ multiplying $1/[\text{bound}]$ is a dilution correction which is necessary since the total site concentration changes as each aliquot of phloretin solution is added to the

cuvette (see Materials and Methods). A correction (<2%) has also been made for the scattering of light by vesicles. The line in Fig. 2 *b* has a linear correlation coefficient of 0.996; the calculated dissociation constant, K_D , is 7.8 ± 0.2 (SE) μM and the number of lipid molecules per binding site, L/S , is 4.02 ± 0.06 . The point-by-point calculated concentration values for all the species required to generate the binding curve shown in Fig. 2 *b* are listed in Table I. The linearity of the binding curve implies that the binding of phloretin to PC vesicles can be described by a single set of independent, equivalent binding

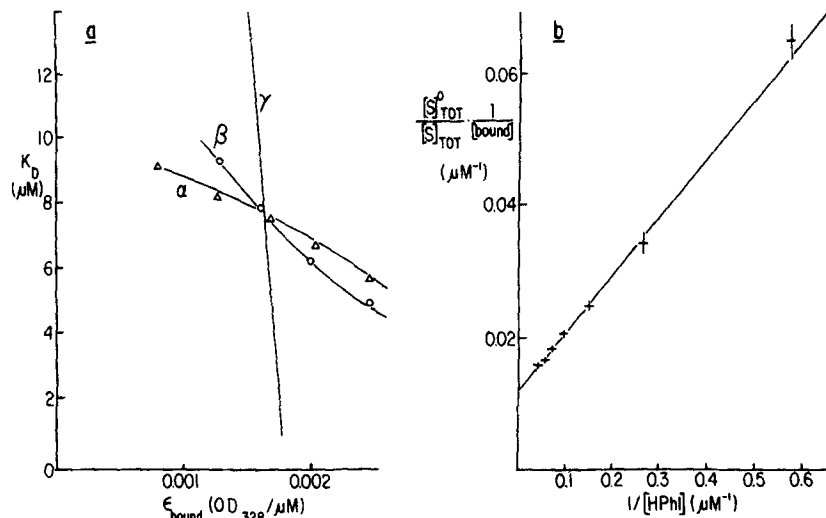


FIGURE 2. Binding data plots. (a). The value of ϵ_{bound} was determined using data from three independent titrations—each using a different PC concentration. Curves α (Δ) and β (\circ) correspond to fitted values of K_D assuming trial values for ϵ_{bound} ; line γ is calculated using Eq. 7. The unique intersection indicates $\epsilon_{\text{bound}} = 0.0016$ and $K_D = 8.0 \mu\text{M}$. (b). Data points from Table I are plotted for the binding of phloretin to PC vesicles at pH 7.30 in one experiment typical of four. Error bars represent 1 standard deviation (SD) from the mean. The y-intercept is $1/[S]_{\text{tot}}$; the slope is $K_D/[S]_{\text{tot}}$ as in Eq. 5. The fitted line has a linear correlation coefficient of 0.996. Thus, the plot in *a* gives ϵ_{bound} and K_D , and the intercept in *b* gives $[S]_{\text{tot}}$. L/S is calculated from $[S]_{\text{tot}}$ and the known concentration of lipid.

sites. A single class of sites has also been found for phloretin binding to red blood cell lipid extracts (Jennings and Solomon, 1976) and for phloretin adsorption onto a planar bilayer (De Levie et al., 1979) which is consistent with the suggestion that phloretin binds almost exclusively to the dipolar region of each membrane surface (Andersen et al., 1976).

VISCOSITY DEPENDENCE The effect of buffer viscosity upon the measured values of K_D and L/S was investigated. Titrations were performed using buffers whose viscosity was set by adding 0–20% glycerol or 0–40% sucrose corresponding to viscosities from one to six times that of water alone. The values of K_D and L/S , which were fitted by least squares for each viscosity

tested, are given in Table II *a* and are identical to the value given in the top line in the absence of glycerol or sucrose ($P > 0.4$, Student's *t* test). The lack of effect of buffer viscosity on K_D and L/S shows that there is no specific interaction between the vesicle-phloretin system and sucrose or glycerol since equilibrium binding is a function of thermodynamic potentials alone.

NARINGENIN The naringenin molecule is similar in structure to the phloretin molecule; the molecular structure of both is shown in Fig. 5.

TABLE I
DATA FROM BINDING TITRATION

[HPhl] _{tot}	[PC] _{tot}	[bound]	[HPhl]	1/[HPhl]	$\frac{[S]_{tot}^0}{[S]_{tot}} \cdot 1/[bound]$
μM	μM	μM	μM	μM^{-1}	μM^{-1}
17.0	317	13.6	1.70	0.588	0.0671
31.4	292	24.6	3.67	0.272	0.0350
43.6	271	30.8	6.40	0.156	0.0253
54.2	252	34.7	9.79	0.102	0.0210
63.5	236	36.9	13.3	0.075	0.0185
71.7	222	38.0	16.8	0.059	0.0169
78.9	210	37.8	20.5	0.048	0.0160

TABLE II
EFFECTS OF VISCOSITY AND pH ON EQUILIBRIUM BINDING
OF PHLORETIN

η/η_{H_2O}	pH	K_D	L/S
(a) Effect of viscosity		μM	
1.0	7.30	8.0 ± 0.2	3.9 ± 0.1
0.78 (sucrose)	7.30	7.4 ± 0.5	4.3 ± 0.4
0.55 (glycerol)	7.30	8.5 ± 0.5	4.2 ± 0.3
0.17 (sucrose)	7.30	7.5 ± 0.4	3.6 ± 0.3
(b) Effect of pH*			
1.0	6.50	14.2 ± 3.0	3.5 ± 0.7
1.0	8.30	3.2 ± 1.5	4.0 ± 0.5
1.0	9.30	2.7 ± 1.5	4.5 ± 0.8

* At pH 4.0, K_D has been determined to be $23 \pm 5 \mu M$ using a fluorescence-quenching technique (Verkman, 1980).

Naringenin has a $pK_a = 7.30$ and an absorption spectrum which is very similar to that of phloretin. Binding titrations were performed at pH 7.30 at 328 nm for the naringenin-PC vesicle system and gave $K_D = 24.2 \pm 2.8$ (SE) μM and $L/S = 3.8 \pm 0.5$. The difference in binding affinity between phloretin ($K_D = 7.8 \mu M$) and naringenin for PC vesicles may reflect differences in dipole characteristics and lipid solubilities and hence in the interaction with the dipolar regions of a lipid bilayer.

pH DEPENDENCE The dependence of K_D and L/S on buffer pH for phloretin-PC vesicle binding was determined to obtain results needed later for kinetic calculations. This data is given in Table II *b*. Binding experiments at

higher pH become increasingly difficult to perform because smaller changes in OD must be detected.

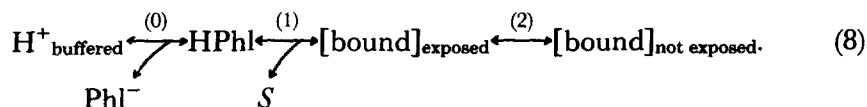
The binding parameters K_D and L/S should be independent of pH provided that the system is described exactly by Eqs. 1 and 2 and that the lipid properties do not vary with pH in the range studied. Though the L/S ratio is independent of pH ($P > 0.3$), the binding affinity increases by a factor of five as the H^+ ion concentration decreases by three orders of magnitude. These data imply that more than 100 [HPhl] molecules bind to a PC vesicle per bound $[Phl^-]$ molecule in the concentration range studied.¹

Temperature-Jump Studies

The temperature of phloretin-PC solutions was jumped by $\sim 4^\circ C$ by a 15,000 V electrical discharge (see Materials and Methods). The concentration of bound phloretin was determined by measuring OD_{328} over times ranging from 0 to 10 ms after the temperature increment occurred. Typical results are shown in Fig. 3. In curve *a*, a temperature jump of 150 μM phloretin alone in 0.1 M Tris plus 0.1 M KCl at pH 7.30 yields a single relaxation process, which is faster ($< 5 \mu s$) than the resolution time of the system. This relaxation process is an acid-base shift in phloretin equilibrium induced by the pH shift of the temperature-dependent Tris buffer and is expected to occur on a submicrosecond time scale (Rose and Stuehr, 1971). In curves *b* and *c*, the addition of 100 and 400 μM concentrations of PC vesicles to the system demonstrates an additional concentration-dependent relaxation process which occurs in tens of microseconds. This process, shown as an increase in OD_{328} with time following the initial upward deflection, corresponds to an increase in $[Phl^-]$ in free solution and a decrease in $[HPhl] + [bound]$. The relaxation time constant of the slow process is altered $< 1\%$ when the 0.1 M Tris buffer is replaced with 0.1 M Tris-maleate, 0.1 M phosphate or 0.1 M morpholinopropane sulfonic acid (MOPS) and is altered $< 2\%$ when a 1 or $2^\circ C$ temperature jump is used instead of the usual $4^\circ C$ jump. This slow process will be shown to be the unbinding process of phloretin from PC vesicles induced by the temperature jump.

The interpretation of the molecular details of binding kinetics requires the choice of an explicit chemical mechanism. Since the equilibrium binding data show that phloretin binds to a single class of noninteracting sites, a simple mechanism to describe the binding process is,

¹ The argument for this conclusion rests on the observation that only a single class of binding sites was found over the pH range studied. The optical density of bound phloretin determined by the method in Fig 2 *a*, $\epsilon_{bound} = 0.0016$, did not vary with pH ($P < 0.01$) and was identical to the optical density of free [HPhl] determined at very low pH. The binding of less than one part $[Phl^-]$ in 100 [HPhl] molecules was detectable for the titration performed at pH 6.5 since ϵ_{bound} can be resolved within 10% and the OD of $[Phl^-]$ exceeds that of [bound] by more than a factor of 10. No $[Phl^-]$ binding was detected for this titration. These experiments do not rule out a very low affinity, high capacity $[Phl^-]$ binding site with a K_D of 1 mM or greater since only a limited concentration range was studied.



Although Eq. 8 is an accurate equilibrium description of phloretin binding to vesicles, it is not obvious whether Eq. 8 applies when kinetics are studied by a relaxation method. The most general description of the phloretin-PC interactions is a combination of three kinds of processes: solution reactions, the binding reaction, and complex intramembraneous processes as shown in Fig. 4. The solution reactions consist of acid-base phloretin equilibria occurring in the Tris buffer. The binding reaction is the binding of [HPhl] to free sites

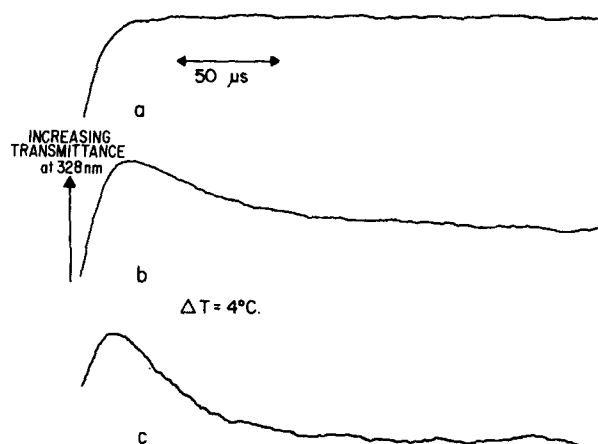


FIGURE 3. Temperature-jump relaxation spectra for phloretin-PC. Typical data for the phloretin-PC system are shown. The negative deflection represents increasing absorption of 328 nm light. Solutions consist of 0.1 M KCl, 0.1 M Tris at pH 7.30 with (a) 150 μM phloretin, (b) 150 μM phloretin + 100 μM PC, and (c) 150 μM phloretin + 400 μM PC.

exposed at the outer vesicle surface. The membrane transfer reactions consist of exchanges of phloretin among free sites present throughout the membrane. Intravesicular processes are neglected since even at the highest phloretin concentrations used, 200 μM , <10% of the vesicles contain one or more phloretin molecules dissolved within the intravesicular space.

Detailed analysis of the problem of determining the effective equilibrium constant for multiple, identical sites which are present on a protein was first considered by Adair (Van Holde, 1971). The present problem differs in two important respects. Firstly, the calculation of Adair does not apply to relaxation kinetics. Secondly, there are many exchange reactions in which a bound phloretin molecule can equilibrate rapidly among vesicle sites present in different locations. As calculated in the Appendix, the mechanism shown in Fig. 4 simplifies exactly to Eq. 8 for the purposes of relaxation calculations.

As Fig. 3 *a* shows, the acid-base reaction (reaction 0 in Eq. 8) is fast compared with the binding reaction (1). Exchange reaction (2) should give rise to a process which is independent of the external phloretin concentration and would correspond to passage over an intramembrane barrier. Reaction (2) has been observed using the temperature-jump and stopped-flow methods² and has a relaxation time constant of ~ 1 s.

Thus, the slower process in Fig. 3 corresponds to the binding process, and for small perturbations, its time constant, τ , is related to the equilibrium concentration of free phloretin $[HPhl]$, free sites $[\bar{S}]$, and pH by Eq. 9 (see

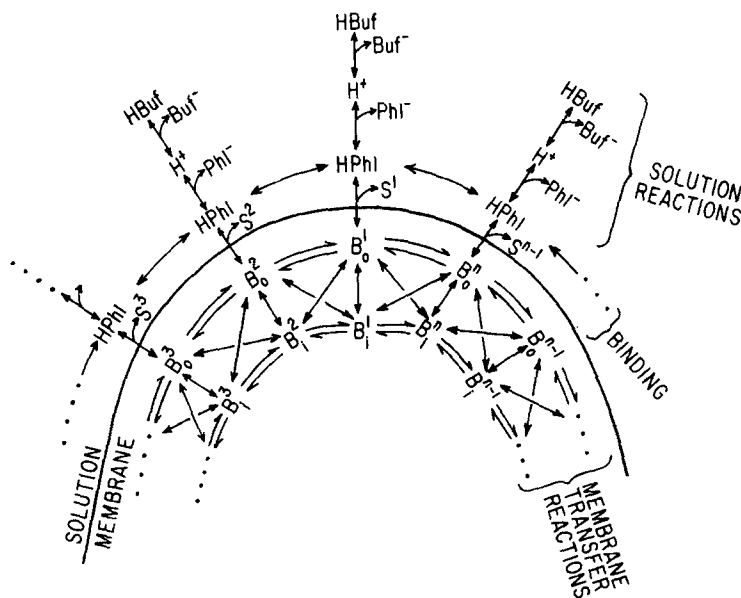


FIGURE 4. Schematic of phloretin-vesicle interactions. Notation: S^i = i th site; B_o^i = i th exposed site; B_i^i = i th nonexposed site. The reaction components consist of HBuf, Buf⁻, H⁺, HPhl, and multiple free sites S^i and bound sites, B_o^i and B_i^i .

Appendix for derivation and assumptions),

$$\frac{1}{\tau} = k_1 \left(\frac{[HPhl]}{[H^+]} + \frac{f_o [\bar{S}] [H^+]}{[H^+] + 10^{-7.3}} \right) + k_{-1}, \quad (9)$$

where f_o is the fraction of total binding sites exposed at the vesicle surface for immediate binding, k_1 is the forward binding rate constant for step 1 of Eq. 8 and k_{-1} is the unbinding rate constant. Fig. 5 shows a plot of τ^{-1} vs. the bracketed quantity in Eq. 9. At pH 7.30, the forward rate constant $k_1 = (2.66 \pm 0.36) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and backward rate constant $k_{-1} = 2,864 \pm 750 \text{ s}^{-1}$. The fraction of exposed sites, f_o which was used for this fit is 0.66.

² Verkman, A. S., and A. K. Solomon. Manuscript in preparation.

The values of k_1 , k_{-1} , and f_o merit further discussion. The factor f_o represents the fraction of total binding sites which are accessible at the outer surface of the membrane. The value of f_o would be expected to approximately equal the fraction of lipid molecules at the external vesicle surface determined by Rothman and Dawidowicz (1975) as 0.7. We first attempted to determine f_o by fitting the parameters k_1 , k_{-1} , and f_o in Eq. 9 to the phloretin data plotted in Fig. 5. The best value for f_o was 0.45 but the 95% confidence interval ranged from 0.2 to 0.85 since the quality of the fit depends only weakly upon f_o . A direct method to determine f_o uses the stopped-flow technique.² In this

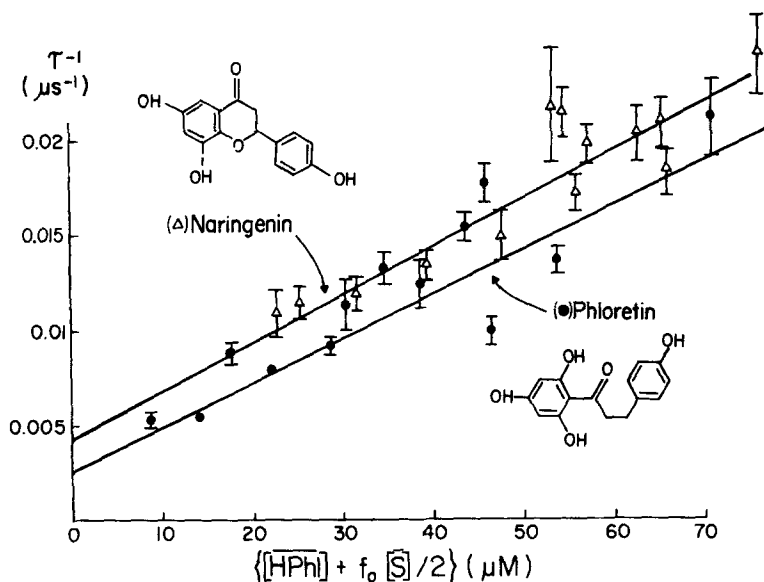


FIGURE 5. Phloretin and naringenin binding kinetics. The temperature-jump relaxation time constants τ^{-1} are plotted against the calculated concentration function, $[\tilde{S}] + f_o [\text{HPhI}]/2$ in Eq. 9 for phloretin and naringenin. The value 2 dividing $f_o [\text{HPhI}]$ is calculated from Eq. 9 with pH = 7.30. Each data point represents the weighted average of fitted time constants for four experiments; error bars are 1 SD from the mean.

technique, phloretin is mixed with vesicles within 10 ms. Two relaxation components are observed: a submillisecond component due to binding of phloretin to external sites and a slower (~ 1 s) component owing to exchange between sites at the external and internal surfaces. An analysis of the amplitudes of these processes yielded $f_o = 0.66 \pm 0.05$.

The forward rate constant k_1 reflects the rate of bimolecular collisions which are successful in binding. Its value may be limited by phloretin diffusion in the external buffer or by the shape of the energy barrier which must be overcome for a successful binding event. For diffusion-limited kinetics, k_1 may be expressed in terms of the diffusion coefficient of phloretin (D), the lipid/site ratio (L/S), the vesicle radius (r_o), Avogadro's number (N_A), and the

number of lipids per vesicle,

$$k_1 = \frac{(4\pi \times 10^{-3}) r_o D N_A (L/S)}{f_o (\text{lipids/vesicle})}. \quad (10)$$

Eq. 10 is derived by equating the chemical kinetic expression,

$$\frac{d[\text{bound}]}{dt} = k_1 f_o [\text{HPhl}][S], \quad (11)$$

with the expression involving the flux, J_{vesicle} , to individual vesicles,

$$\frac{d[\text{bound}]}{dt} = J_{\text{vesicle}} \cdot (\text{number of vesicles}), \quad (12)$$

where $J_{\text{vesicle}} = (4\pi \times 10^{-3}) r_o D [\text{HPhl}]$ (Berg and Purcell, 1977), and the number of vesicles is $[S] N_A (L/S) / (\text{lipids/vesicle})$. This formula is valid for concentrations expressed in molar, and for r_o and D expressed in centimeter-gram-second units. Substituting appropriate values [$k_1^{\text{HPhl}} = 1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ under conditions when $[\text{Phl}^-]$ is negligible (see Eq. 14); $L/S = 4.0$ and $f_o = 0.66$ obtained experimentally, and the published values $r_o = 100 \text{ \AA}$, lipids/vesicle = 2,700 (Huang, 1969)] D is calculated to be $0.8 \times 10^{-5} \text{ cm}^2/\text{s}$. Clark presents an empirical relation for the diffusion coefficient of a series of organic molecules in water as a function of molecular weight (Clark, 1952). The empirical value of D is $0.5 \times 10^{-5} \text{ cm}^2/\text{s}$ for a molecule having the same molecular weight as phloretin (274 daltons). This empirical value agrees reasonably well with the calculated value of D for diffusion limited kinetics.

Steady-state conditions are implicit in the derivation of Eq. 10 because J_{vesicle} is taken as constant. Although the relaxation process perturbs the system from one steady state to another, the steady-state assumption may still be used, providing the time required to establish the new steady state is fast compared to the reaction being studied. Amdur and Hammes (1966) consider the time dependence to be negligible³ at times greater than $\sim 10^{-7} \text{ s}$, whereas the phloretin binding has a relaxation time of greater than 10^{-5} s .

The observed unbinding rate, k_{-1} depends upon D because the movement of a phloretin molecule from the vesicle surface to a position far from the vesicle in solution depends both on the intrinsic unbinding rate and upon diffusion away from the surface. In general, when k_1 is diffusion limited, k_{-1} will also be diffusion limited and therefore proportional to D (Amdur and Hammes, 1966; Schurr, 1970). In the diffusion-limited case, the rate at which molecules leave a vesicle surface is proportional to $D(d[\text{HPhl}]/dr)$ by Fick's Law. The steady-state concentration profile for phloretin unbinding from a sphere of radius r_o , and surface phloretin concentration $[\text{HPhl}]_s$, is independent of D , that is: $[\text{HPhl}(r)] = [\text{HPhl}]_s (r_o/r)$. Consequently, the observed unbinding rate is proportional to D . The height of the interfacial barrier is reflected in $[\text{HPhl}]_s$ which, as shown elsewhere (Amdur and Hammes, 1966;

³ The steady-state assumption is discussed in Amdur and Hammes (1966) and a complete study of the effect of the time-dependent term has been given by Schurr (1970).

Verkman, 1979) is given by

$$[\text{HPhl}]_s = \frac{[\text{HPhl}]_s^{\text{no potential}}}{\int_{r_0}^{\infty} [e^{v(r)/r^2}] dr}, \quad (13)$$

where $v(r)$ is the interfacial potential barrier. The binding process is governed by D because the interfacial potential barrier is small under our experimental conditions (see Verkman, 1979) so that the surface phloretin concentration, $[\text{HPhl}]_s$, is nearly equal to $[\text{HPhl}]_s^{\text{no potential}}$.

The ratio k_{-1}/k_1 , the equilibrium dissociation constant, is independent of D and hence of solution viscosity (see also Amdur and Hammes, 1966; and Schurr, 1970). It is calculated to be $10.7 \pm 3.1 \mu\text{M}$. This result, computed from kinetics studies, agrees with the value of $8.0 \pm 0.2 \mu\text{M}$, determined from equilibrium measurements and given in the first line of Table II.

Characterization of the Binding Process

Three additional experiments will now be described which further characterize the diffusion-limited binding process. The dependence of binding rate on viscosity will demonstrate that the observed binding is a bimolecular process consistent with a diffusion-limited process. The phloretin analogue, naringenin, will be shown to have the same binding kinetics as those of phloretin, which suggests that diffusion rather than a specific interfacial barrier limits the binding rate. Finally, the dependence of binding rate on pH will support the model proposed for the binding mechanism and indicate that binding is diffusion limited.

EFFECT OF VISCOSITY ON BINDING KINETICS Temperature-jump experiments on the phloretin-PC system in several buffers of different viscosities were performed at pH 7.30. If the observed concentration-dependent relaxation process is indeed diffusion-limited binding, k_1 is given by Eq. 10 and depends upon viscosity through the diffusion coefficient, D , which is inversely proportional to viscosity by the Stokes-Einstein relation. The data in Fig. 6 show that $k_1 \propto 1/\eta$ since they are fitted by a straight line passing through the origin ($r = 0.95$). This is consistent with the expected dependence for diffusion-limited processes. As has already been pointed out, k_{-1} is dependent upon D , so that the dissociation constant, K_D , should be independent of viscosity as Table II shows.

NARINGENIN BINDING KINETICS The binding kinetics for the naringenin-PC vesicle system were studied by the same procedures used for phloretin and the results are also plotted in Fig. 5. The slope, or binding rate constant, for naringenin binding is $(2.93 \pm 0.34) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$; for phloretin binding it is $(2.66 \pm 0.36) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. The intercept, or unbinding rate constant for naringenin unbinding, is $4,189 \pm 1,000 \text{ s}^{-1}$; for phloretin unbinding it is $2,864 \pm 750 \text{ s}^{-1}$.

Since naringenin and phloretin have similar chemical structures and molecular weights, they should have comparable diffusion coefficients in buffer.

Therefore, Eq. 10 would imply that both molecules should have equivalent binding rate constants if diffusion is rate limiting, which would be the case if the effective potential barrier for binding could be neglected compared with that for diffusion. The binding rate constants for phloretin and naringenin are identical ($P > 0.2$). However, the unbinding rate constants should differ since the equilibrium affinity for PC vesicles is $8 \mu\text{M}$ for phloretin and $24 \mu\text{M}$ for naringenin. This observation implies that the potential barrier for unbinding may not be neglected compared with that for diffusion so that constraints introduced by both processes are contained within the apparent unbinding constant (see Verkman, 1979). The unbinding rate constants are determined

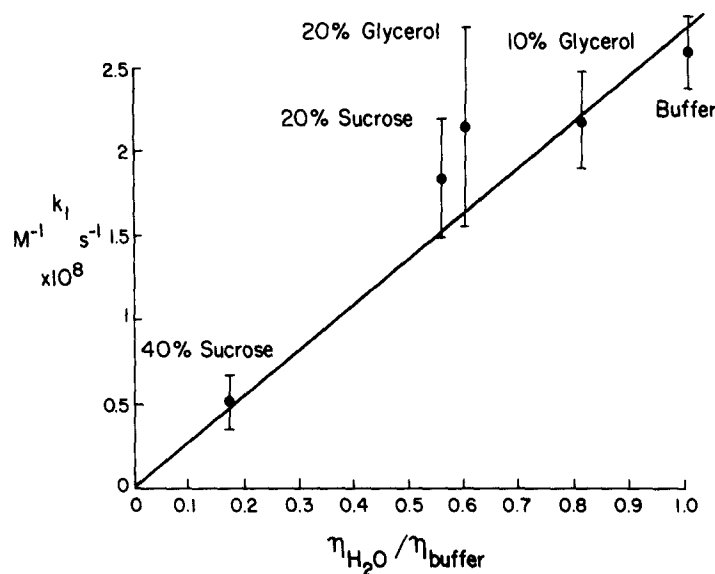


FIGURE 6. Viscosity dependence of k_1 . Reciprocal viscosity is plotted against the binding rate constant k_1 . The least squares line shows that $k_1 \propto 1/\text{viscosity}$. Each point corresponds to five experiments performed on the phloretin-PC vesicle system in buffers whose viscosities are changed by addition of glycerol or sucrose.

by the intercept of the lines in Fig. 5 and are very difficult to determine exactly as shown by the relatively large standard deviations for the k_{-1} values. Nonetheless, the k_{-1} values for phloretin and naringenin differ significantly ($P < 0.01$), which is consistent with a diffusion-limited binding process and an unbinding process, which is dependent not only on specific molecular structure, but also on the diffusion coefficient.

EFFECT OF pH ON BINDING KINETICS The kinetics of phloretin binding to PC vesicles at several pH values are shown in Fig. 7. Solutions were prepared by adding known quantities of PC vesicles and phloretin to buffers set at

selected pH in the range of 6.5–9.3. The abscissa in Fig. 7 was calculated by using equilibrium binding constants from Table II and the ordinate was determined from temperature-jump experiments whose relaxation time constants were determined by the method described in a previous paper (Verkman et al., 1979).

Table III and Fig. 7 demonstrate the striking increase in the binding rate constant, k_1 , with increasing pH. This effect is similar to the enhanced permeability of weak acids across lipid bilayer membranes (Gutknecht and Tosteson, 1973). It can be understood qualitatively as follows: with increasing

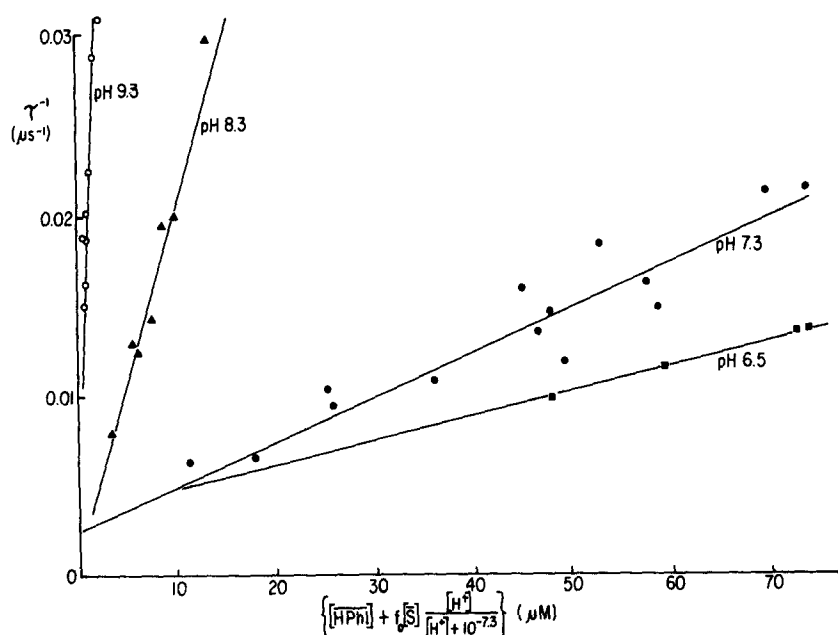


FIGURE 7. Data obtained from relaxation experiments for the phloretin-PC vesicle system as a function of pH are plotted as in Fig. 5. The lines have been fitted by least squares and the correlation coefficients range from 0.93 to 0.98.

pH, a greater fraction of phloretin is in its ionized form $[Phl^-]$. If the phloretin acid-base equilibrium is fast compared with diffusion, the total inward phloretin flux toward a vesicle, which is proportional to the binding rate, will consist of a component from $[HPhl]$ alone plus an additional enhancement due to $[HPhl]$ made rapidly available near the membrane surface by acid-base shift.

Quantitatively, the problem can be formulated in terms of coupled diffusion equations with appropriate boundary conditions as outlined in the appendix. These equations show that in the limit when the equilibrium $HPhl \rightleftharpoons Phl^- + H^+$ is fast compared with diffusion,

$$k_1 = k_1^{\text{HPhl}} \left(1 + \frac{[\text{Phl}^-]}{[\text{HPhl}]} \right), \quad (14)$$

where k_1^{HPhl} would be the observed binding rate constant if only HPhl were present as under conditions at very low pH. A straight line fit to the data in Table III yields $k_1^{\text{HPhl}} = (1.6 \pm 0.3) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$; an intercept of $(0.26 \pm 0.74) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $r = 0.96$. This implies that the submicrosecond phloretin equilibration is fast enough to enhance observed binding maximally. These experiments confirm that phloretin binding to PC vesicles is diffusion limited and demonstrate that the equations and model developed for phloretin-PC binding kinetics are valid over a 10^3 -fold $[\text{Phl}^-]/[\text{HPhl}]$ concentration ratio.⁴

A detailed molecular description of nonelectrolyte permeation across membranes, either in model systems or in living cells, depends upon an understanding of the kinetics of each individual step. The temperature-jump technique in conjunction with equilibrium binding measurements, has made it possible to measure the rates of binding and unbinding of phloretin to PC vesicles. Though the binding process is diffusion limited, the unbinding process de-

TABLE III
EFFECT OF BUFFER pH ON k_1

pH	$1 + [\text{Phl}^-]/[\text{HPhl}]$	$k_1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$
6.5	1.16	1.4 ± 0.2
7.3	2.0	2.7 ± 0.4
8.3	11.0	11 ± 1.6
9.3	101.0	110 ± 13

pends upon the energy barriers at the membrane-solution interface. The temperature-jump technique should be suitable for further measurement of the entropy and enthalpy of the interface reaction and would thus provide a sound basis for studies of adjacent steps in the transport process.

APPENDIX

Derivation of Eq. 9

For reaction (2), slow compared with reaction (1) in Eq. 8, we may write,

$$\frac{d[b]}{dt} = k_1[\text{HPhl}][S] - k_{-1}[b]; \quad (\text{A-1})$$

$$\frac{d[\text{Phl}^-]}{dt} = -k_0[\text{H}^+][\text{Phl}^-] + k_{-0}[\text{HPhl}], \quad (\text{A-2})$$

where $[b] = [\text{bound}]_{\text{exposed}}$, and k_0 and k_{-0} are forward and reverse rate constants for reaction (0) in Eq. 8.

⁴ Although the equilibrium binding affinities shown in Table II depend upon pH, the binding rates are relatively insensitive to the value of K_D . The calculated values of k_1 for a single pH, using Eq. 9, change by less than 40% for the fivefold change in K_D over the concentration range studied.

When the system is perturbed the new concentrations are,

$$\begin{aligned} [b] &\rightarrow [\bar{b}] + x \\ [S] &\rightarrow [\bar{S}] - x \\ [\text{Phl}^-] &\rightarrow [\bar{\text{Phl}}^-] - y \\ [\text{HPhl}] &\rightarrow [\bar{\text{HPhl}}] - x + y. \end{aligned} \quad (\text{A-3})$$

By substituting conditions (A-1) in (A-2) and (A-3), neglecting second-order terms (e.g., xy), using the fact that (A-1) is much slower than (A-2), $dy/dt = 0$, and applying conditions (A-4),

$$\begin{aligned} k_1[\bar{\text{HPhl}}][\bar{S}] &= k_{-1}[\bar{b}] \\ k_0[\bar{\text{H}^+}][\bar{\text{Phl}}^-] &= k_{-0}[\bar{\text{HPhl}}], \end{aligned} \quad (\text{A-4})$$

we get

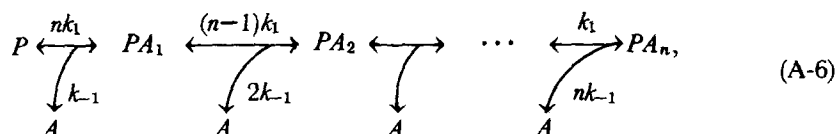
$$-\frac{dx}{dt} = x \left[k_1 \left([\bar{\text{HPhl}}] + f_0[\bar{S}] \frac{[\bar{\text{H}^+}]}{[\bar{\text{H}^+}] + K_D} \right) + k_{-1} \right], \quad (\text{A-5})$$

where $K_D = k_{-0}/k_0$ and $f_0[\bar{S}]$ represents the concentration of exposed sites. This treatment makes no allowance for the pH dependence of k_1 and k_{-1} , and hence is valid at only a single pH.

Simplification of Mechanism Shown in Fig. 4

The purpose of this section of the Appendix is to show that a perturbation of the complex reaction scheme illustrated in Fig. 4 uncouples into the problem of a perturbation of the scheme in Eq. 8.

(a) Let us first consider the reaction scheme for the binding of a substrate A to a vesicle P having n independent sites,



where the rate constants are derived from simple combinatorics (Van Holde, 1971) and are shown accompanying the arrows above. The kinetic expression corresponding to the above scheme is

$$-\frac{d[A]}{dt} = k_1 \left([A]n[P] + \sum_{i=1}^n (n-i)[PA_i] \right) - k_{-1} \sum_{i=1}^n i[PA_i]. \quad (\text{A-7})$$

By using conservation, applying the perturbation,

$$\begin{aligned} [PA_i] &\rightarrow [\bar{PA}_i] + x_i \\ [A] &\rightarrow [\bar{A}] - \sum_1^n ix_i \\ [P] &\rightarrow [\bar{P}] - \sum_1^n x_i, \end{aligned} \quad (\text{A-8})$$

and neglecting second-order terms $x_i x_j$,

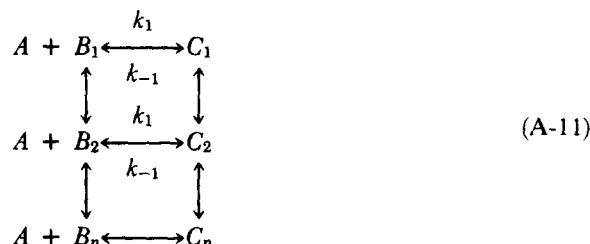
$$-\frac{dr}{dt} = k_1[\bar{A}]\zeta + k_1\zeta\left(n[\bar{P}] + \sum_{i=1}^n (n-i)[\bar{PA}_i]\right) + k_{-1}\zeta, \quad (\text{A-9})$$

where $\zeta = \sum_{i=1}^n (-ix_i)$, which implies

$$\frac{1}{\tau} = k_1\left\{[\bar{A}] + \left(n[\bar{P}] + \sum_{i=1}^n (n-i)[\bar{PA}_i]\right)\right\} + k_{-1}. \quad (\text{A-10})$$

The bracketed term is simply the sum of equilibrium concentration of substrate A and of free sites. This implies that, if binding sites are independent, there is no difference in the perturbation relaxation spectrum whether the sites are confined to a particular geometry (vesicle) or are free in solution.

(b) Consider the parallel reaction scheme,



By using the kinetic expression,

$$-\frac{d[A]}{dt} = k_1[A] \sum_{i=1}^n [B_i] - k_{-1} \sum_{i=1}^n [C_i], \quad (\text{A-12})$$

and applying the perturbation,

$$\begin{aligned} [A] &\rightarrow [\bar{A}] + \sum x_i \\ [B_i] &\rightarrow [\bar{B}_i] + x_i + y_i; \quad \sum y_i = 0 \\ [C_i] &\rightarrow [\bar{C}_i] + z_i - x_i; \quad \sum z_i = 0, \end{aligned} \quad (\text{A-13})$$

we find

$$-\frac{d(\sum x_i)}{dt} = k_1\{[\bar{A}] \sum (x_i + y_i) + \sum [\bar{B}_i] \sum x_i\} + k_{-1} \sum (x_i - z_i), \quad (\text{A-14})$$

which gives

$$\frac{1}{\tau} = k_1\{[\bar{A}] + \sum_{i=1}^n [\bar{B}_i]\} + k_{-1}. \quad (\text{A-15})$$

This result shows that equivalent parallel reactions reveal the same relaxation spectra as each individual reaction.

Calculations *a* and *b* imply that the system in Fig. 4 uncouples into the scheme in Eq. 8 because the complex scheme consists precisely of parallel equivalent reactions confined to a vesicle.

Derivation of Eq. 14

We wish to calculate an expression for the rate of [HPhl] binding to a sphere of radius, r , with the solution acid-base equilibrium, $\text{HPhl} \leftrightarrow \text{Phl}^- + \text{H}^+$, which is described by

the kinetic expression,

$$\frac{d[\text{HPhl}]}{dt} = k_0[\text{H}^+][\text{Phl}^-] - k_{-0}[\text{HPhl}], \quad (\text{A-16})$$

where k_0 and k_{-0} are forward and reverse rate constants, respectively. The local flux of a molecule i ($[\text{HPhl}]$ or $[\text{Phl}^-]$), J_i , is given by the Fick relation,

$$J_i = -D_i \frac{\partial [i]}{\partial r}. \quad (\text{A-17})$$

Local particle continuity demands

$$\frac{d[\text{HPhl}]}{dt} = k_0[\text{H}^+][\text{Phl}^-] - k_{-0}[\text{HPhl}] - \frac{\partial J_{\text{HPhl}}}{\partial r}. \quad (\text{A-18})$$

This implies that in steady state³ the coupled system of differential equations, where $\text{Phl}^-(r)$ and $\text{HPhl}(r)$ are position-dependent concentrations,

$$\begin{aligned} k_0[\text{H}^+]\text{Phl}^-(r) - k_{-0}\text{HPhl}(r) + D_{\text{HPhl}}\nabla^2\text{HPhl}(r) &= 0 \\ k_0[\text{H}^+]\text{Phl}^-(r) - k_{-0}\text{HPhl}(r) - D_{\text{Phl}^-}\nabla^2\text{Phl}^-(r) &= 0, \end{aligned} \quad (\text{A-19})$$

and subject to the boundary conditions $\text{HPhl}(a) = 0$, $\text{HPhl}(\infty) = [\text{HPhl}]_o$, $(d/dr)\text{Phl}^-(a) = 0$, and $\text{Phl}^-(\infty) = [\text{Phl}^-]_o$, describe the binding process for $[\text{HPhl}]$ to a sphere of radius a . The desired binding flux, J_{binding} , is then

$$J_{\text{binding}} = 4\pi a^2 [J_{\text{HPhl}}(a) + J_{\text{Phl}^-}(a)], \quad (\text{A-20})$$

which solves the problem in principle. In the limit in which acid-base equilibration is fast compared with diffusion, $k_0[\text{H}^+][\text{Phl}^-] = k_{-0}[\text{HPhl}]$ and assuming $D_{\text{HPhl}} = D_{\text{Phl}^-}$, the enhancement of k_1 , $[J_{\text{HPhl}}(a) + J_{\text{Phl}^-}(a)]/J_{\text{HPhl}}(a)$, due to the presence of Phl^- , becomes $1 + ([\text{Phl}^-]/[\text{HPhl}])$, using Eqs. A-17 and A-19.

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